Cardiolipin metabolism in Barth syndrome
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Identification and characterization of human cardiolipin synthase

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Abstract
The mitochondrial phospholipid cardiolipin is synthesized from cytidinediphosphate-diacylglycerol and phosphatidylglycerol, a process catalyzed by the enzyme cardiolipin synthase. In this study we identified a human candidate gene/cDNA for cardiolipin synthase, C20orf155. Expression of this candidate cDNA in the (cardiolipin synthase-deficient) crd1Δ yeast confirmed that it indeed encodes human cardiolipin synthase. Purified mitochondria of the crd1Δ expressing human cardiolipin synthase were used to characterize the enzyme. It has an alkaline pH optimum, requires divalent cations for activity and appears to have a different substrate preference for cytidinediphosphate-diacylglycerol species when compared to phosphatidylglycerol species. The possible implications for CL synthesis and remodeling are discussed.

Abbreviations
At, Arabidopsis thaliana
BTP, Bis-tris propane
CDP-DAG, cytidinediphosphate-diacylglycerol
CL, cardiolipin
CLS, cardiolipin synthase
Hs, Homo sapiens
PG, phosphatidylglycerol
Sc, Saccharomyces cerevisiae
Introduction
Cardiolipin (CL) is an anionic phospholipid with a unique diphosphatidylglycerol structure, containing four acyl groups [1]. In eukaryotes, CL is predominantly found in the inner mitochondrial membrane where it comprises approximately 16% of total phospholipids [2]. It has been shown that CL is essential for maintaining the mitochondrial membrane potential [3], is involved in the stabilization of supercomplexes of respiratory chain complexes III and IV [4] and is required for the activity of several mitochondrial enzymes, e.g. the ADP/ATP carrier [5]. More recent reports show that CL and its metabolites are also involved in apoptosome mediated apoptosis [6].

Synthesis of CL occurs at the inner mitochondrial membrane by the transfer of the phosphatidyl group of cytidinediphosphate-diacylglycerol (CDP-DAG) to phosphatidylglycerol (PG), a process catalyzed by the enzyme CL synthase (CLS). This conversion of PG into CL is so effective that mitochondrial membranes only contain trace amounts of PG [7]. CLS from rat liver [8,9], yeast [10-13], and plants [14,15] have been characterized and the respective genes of yeast and Arabidopsis thaliana were cloned [10,11,13,15,16]. In general, mitochondrial CLSs have an alkaline pH optimum, require divalent cations (Co²⁺, Mg²⁺, or Mn²⁺) for activity and possess distinctly lower Kₘ values for CDP-DAG than for PG [9]. CLS from rat liver mitochondria displays the highest activity towards medium-chain CDP-DAGs (dilauroyl and dimyristoyl) when compared to long-chain CDP-DAGs (dipalmitoyl and distearoyl) and has a preference for unsaturated long-chain CDP-DAG species (dioleoyl = dilinoleoyl > distearoyl) but it is more active with CDP-1-palmitoyl, 2-oleoylglycerol than with CDP-dioleoylglycerol or –dilinoleoylglycerol [8]. Hence, according to the substrate species specificity the CLS cannot establish the typical fatty acid pattern of CL in mammalian cells, predominantly consisting of linoleic acid. This typical fatty acid composition is created by remodeling of newly synthesized CL. It is generally accepted that at least two CL remodeling systems exist in mammalian cells which are involved in obtaining the observed CL composition. CL has been shown to be remodeled by transacylation from phosphatidylcholine and phosphatidylethanolamine [17] and by deacylation/reacylation [18]. Impaired remodeling of CL in human cells caused by a defect in the tafazzin gene is associated with Barth syndrome, a severe genetic disorder characterized by cardioskeletal myopathy, neutropenia and respiratory chain defects [19-21]. In this study we aimed to identify and characterize the human CLS because the substrate preference of CLS has important implications for remodeling of de novo synthesized CL.

Materials and Methods
Cloning and expression of the human candidate CLS cDNA in Saccharomyces cerevisiae
The putative human CLS (HsCLS) was identified by sequence homology searches using the Swiss Institute of Bioinformatics BLAST network service (http://www.expasy.org/) [22] employing the CLS amino acid sequences of yeast (ScCLS or crd1 or YDL142c) and Arabidopsis thaliana (AtCLS or At4g04870). The HsCLS candidate cDNA sequence was amplified by PCR from I.M.A.G.E. clone 2011896 (ATCC, Manassas, VA), using a forward primer 5’- GGT ACC ATG CTA GCC TTG CGC GTG GC -3’ containing a KpnI restriction site and a reverse primer 5’- GTC GAC
TCA GTC TTT TAT CAC CTG AAC AGT C -3' containing a SalI restriction site. A second reverse primer, 5'- GTC GAC TCA ATG ATG ATG ATG ATG GTC TTT TAT CAC CTG AAC AGT C -3' containing a SalI restriction site was used to amplify the open reading frame including a 3'-sequence encoding a C-terminal 6 × HIS-tag. Both PCR products were ligated into the pGEM-T vector (Promega, Madison, WI, USA). Sequence analysis was performed to exclude sequence errors introduced by the Taq polymerase, and both the tagged and untagged cDNAs were cloned into the KpnI and SalI restriction sites of the pYPGK18 vector suitable for constitutive expression in yeast [20]. Both constructs were transformed to the crd1Δ (BY4742; MATalpha; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YDL142c::kanMX4) and to the BY4742 wild type (BY4742; MATalpha; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) yeast strain using the lithium acetate procedure [23]. Both yeast strains were a kind gift of Dr. Toon de Kroon, Utrecht University. Yeast cells were grown at 28°C on minimal glucose medium, containing 0.67% (w/v) Yeast Nitrogen Base without amino acids (Difco Sparks, MD, USA) and 2% (w/v) glucose (Sigma, St. Louis, MO, USA), containing the appropriate amino acids required for growth.

**Compensation analysis**

For growth phenotype compensation analysis, transformants were grown in minimal glucose medium and harvested in mid-exponential phase. Cells were washed in phosphate-buffered saline and resuspended to OD_{600}=1. Subsequently, the yeast suspension was diluted 10^1, 10^2, 10^3, and 10^4 times, and 6 µl of each dilution was spotted on minimal glucose plates (0.67% (w/v) Yeast Nitrogen Base without amino acids, 2% (w/v) glucose and 2% (w/v) agar) which were placed at 37°C.

To analyze the PG and CL content in the different transformants, 50 ml yeast culture was harvested in mid-exponential phase and pelleted. Cell pellets were lyophilized overnight, after which lipids were extracted. Ten milligrams of lyophilized yeast powder were resuspended in 1 ml of methanol and sonicated for 1 minute at 9 W using a tip sonicator. Two milliliters of chloroform were added followed by sonication for 20 minutes in a sonicator bath. After addition of 1 ml of demineralized water the internal standard (0.4 nmol of CL(14:0)₄ (Avanti Polar Lipids, Alabaster, AL) dissolved in 50 µl chloroform) was added to the extraction. This mixture was shaken vigorously and placed on ice for 15 min, after which it was centrifuged at 1000 × g for 10 min. The organic layer was transferred into another tube, and the aqueous layer was reextracted with 3 ml of 2:1 chloroform-methanol (v/v). The combined organic layers were evaporated under a stream of nitrogen at 45°C. The residue was dissolved in 150 µl of chloroform/methanol/water (50:45:5 v/v/v) containing 0.01% NH₄OH, and 5 µl of this solution was injected into the HPLC MS system.

**HPLC Mass spectrometry**

The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller and an autosampler (Thermo Electron Corporation, Waltham, MA). The column temperature was maintained at 25°C. The lipid extract was injected onto a 2.1×250 mm silica column, 5 µm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform-methanol, 97:3, v/v) and solution A (methanol-water, 85:15, v/v). Solution A and B contained 0.1 ml and
0.01 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: 0–10 min: 20% A to 100% A; 10-12 min, 100% A; 12-12.1 min: 100% A to 0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 µl/min eluent was introduced into the mass spectrometer.

A TSQ Quantum AM (Thermo Electron Corporation) was used in the negative electrospray ionization mode. Nitrogen was used as nebulizing gas. The source collision-induced dissociation collision energy was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300°C. Mass spectra of CL molecular species were obtained by continuous scanning from m/z 400 to m/z 1000 with a scan time of 2 s. PG molecular species were measured using a parent ion scan (from m/z 400 to m/z 1000) for m/z 172.1 in the positive ion mode. The respective spectra of CL and PG species were acquired during their corresponding retention time in the HPLC elution profile. A summation of the peak areas of the complete isotopic clusters of the different CL or PG species were divided by the peak area of the whole isotopic cluster of the corresponding internal standard. This ratio was used as a measure for the total amount of CL and PG. Levels of the wild type BY4742 strain were set at 100%.

Isolation of yeast mitochondria
The crd1Δ yeast strain, and the crd1Δ expressing the putative HsCLS were grown in 5 liter flasks containing 1.5 liters of 2% glucose medium at 28°C (225 rpm). Cells were harvested by centrifugation at 1900 ×g and washed with demineralized water. Spheroplasts were prepared using the zymolyase procedure [24]. Yeast mitochondria were isolated as described previously [25]. The final mitochondrial pellet was resuspended in buffer containing 10 mM Tris-maleate, 0.6 M mannitol, and 0.5 mM phosphate, pH 6.8. Protein quantification of the mitochondrial fraction was performed as described by Bradford [26]. Mitochondria were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

CLS enzyme activity measurement and HsCLS characterization
For CLS assays, isolated mitochondria were washed twice with 10 mM Bis-tris propane-HCl (BTP) buffer pH 7.4, and resuspended in this buffer containing 50% glycerol (about 1 mg protein/ml). One to 6 µg of mitochondrial protein was used for CLS assays in a total volume of 50 µl, as described before [15], but the reaction mixtures were incubated at 37°C for 1 h. The pH optimum was determined using 4 µM CDP-dioleoyl[U-14C]-glycerol and 1 mM dioleoyl-PG in a reaction mixture containing 50 mM BTP-HCl buffer at different pH values. PG substrate specificity of the HsCLS was tested in reaction mixtures containing 50 mM BTP buffer, pH 8.5, 4 µM CDP-dioleoyl[U-14C]-glycerol (330 dpm/pmol), 100 mM CoCl₂ and increasing concentrations of different PG species (dioleoyl-PG, 1-palmitoyl-2-oleoyl-PG, and dipalmitoyl-PG were from Sigma, dilinoleoyl-PG and 1-palmitoyl-2-linoleoyl-PG were from Avanti Polar Lipids). CDP-DAG substrate selectivity was determined by a competition experiment. To this end, CLS activity was measured with 1 mM PG (dioleoyl species) and labeled CDP-dioleoylglycerol (2.6 µM) in the presence of increasing concentrations of different unlabeled CDP-
DAG species (Sigma) and the degree of inhibition in the labeling rates of CL was used as a read-out. Reaction mixtures were stopped and lipids were extracted with 240 µl chloroform:methanol (1:1, v/v) containing 50 µg CL and 100 µl 0.2 M H\textsubscript{3}PO\textsubscript{4}, 1 M KCl. After phase separation, the chloroform layers were analyzed by thin layer chromatography on Silica Gel 60 (Merck) plates in chloroform:methanol:glacial acetic acid (65:25:8, v/v). Radioactively labeled lipids were visualized with a Bioimager FLA 3000 (Raytest, Straubing, Germany) and quantified by scintillation counting.

Results and discussion

Identification of HsCLS and complementation of crd1Δ

To identify the HsCLS gene/cDNA, we performed BLAST analysis using the AtCLS (At4g04870) and ScCLS (crd1 or YDL142c) amino acid sequences and identified a (predicted) human gene termed C20orf155. The translated putative open reading frame showed high sequence homology with both CLS orthologues. No other human protein displayed significant homology towards the confirmed CLS sequences. The putative HsCLS gene is localized at chromosome 20p13-p12.3 and encodes a protein of 301 amino acids. The protein is predicted to contain a C-terminal transmembrane segment (http://psort.ims.u-tokyo.ac.jp) [27] and belongs to the CDP-alcohol phosphatidyltransferase class-I family like the CLS of other eukaryotes [10,11,13,15,16]. It contains a putative N-terminal mitochondrial targeting sequence as predicted by the subcellular localization program MitoProtII (http://ihg.gsf.de/ihg/mitoprot.html) [28]. We amplified the candidate open reading frame by PCR from I.M.A.G.E. clone 2011896 and cloned it into the pYPGK18 vector, suitable for constitutive expression in yeast. The candidate cDNA and the empty vector were transformed to the CLS-deficient, and therefore completely CL-deficient, crd1Δ strain of S. cerevisiae. This deletion mutant displays delayed growth at elevated temperatures [29] and we used this property as a read-out to investigate whether the human candidate cDNA protein product is functionally active as a CLS. Figure 1 shows that expression of the HsCLS candidate cDNA results in complete complementation of the growth phenotype, which is not observed in the CLS-deficient strain transformed with the empty vector (pYPGK18). Like for the untagged HsCLS, expression of the HIS-tagged HsCLS candidate protein restores growth at elevated temperatures (figure 1).

Figure 1. Complementation analysis by growth phenotype.

Yeast suspensions of wild type (WT) strain containing the empty vector pYPGK18, crd1Δ+pYPGK18, crd1Δ+HsCLS and crd1Δ+HsCLS-HIS were diluted 10\textsuperscript{1}, 10\textsuperscript{2}, 10\textsuperscript{3}, and 10\textsuperscript{4} times, spotted on 2% glucose plate and incubated at 37°C. Compared to wild type, crd1Δ clearly shows retarded growth, which is restored by expression of either HsCLS or HsCLS-HIS.
Figure 2. Complementation analysis by CL profile analysis. CL profiles of wild type and the \( \text{crd1} \Delta \) mutant strain containing the empty vector pYPGK18 (WT+pYPGK18, \( \text{crd1} \Delta +\text{pYPGK18} \)) or a HsCLS gene construct (\( \text{crd1} \Delta +\text{HsCLS} \) and \( \text{crd1} \Delta +\text{HsCLS-HIS} \)) are shown in the region from m/z 600 to m/z 720. Peaks at m/z 619.5 (CL i.s.) and m/z 665.5 (PG i.s.) represent the internal standards of CL and PG, respectively. The abundance of the CL internal standard was set at 100% (PG i.s. is not fully depicted). When compared to the wild type yeast, CLs are clearly deficient in \( \text{crd1} \Delta \). Expression of either HsCLS or HsCLS-HIS completely normalizes the CL profile and reduces the PG levels, which are elevated in \( \text{crd1} \Delta \) (arrows).
To investigate whether CL levels were restored in the crd1Δ expressing the HsCLS candidate cDNA, we performed CL and PG analysis by HPLC-MS in the crd1Δ transformed with the putative HsCLS cDNA (with and without C-terminal HIS-tag) and the empty vector (pYPGK18). These CL/PG spectra were compared to the wild type CL/PG spectrum. Figure 2 shows that CLs are present in the transformant expressing the putative HsCLS whereas the deletion mutant transformed with the empty vector completely lacks CL. To compensate for the lack of CL, the crd1Δ strain is known to contain highly elevated levels of PG [10]. A semiquantitative determination of the CL and PG levels in the different transformants is depicted in figure 3. The crd1Δ strain contains over 35 times more PG than the wild type strain. Expression of either HsCLS or HsCLS-HIS almost completely reverses the accumulation of PG, which is most likely used for CL synthesis (figure 3).

**Figure 3. Semiquantitative analysis of CL and PG levels.** CL and PG levels of wild type and the crd1Δ mutant strain containing the empty vector pYPGK18 (WT+pYPGK18, crd1Δ+pYPGK18) or a HsCLS gene construct (crd1Δ+HsCLS and crd1Δ+HsCLS-HIS) were determined by HPLC MS analysis. WT levels were set at 100% for both CL and PG. PG levels are highly elevated (~35×) in crd1Δ whereas CL is undetectable (n.d.). Expression of either HsCLS or HsCLS-HIS completely normalizes the CL profile and reduces the amount of PG to 4-6 times the wild type level.

In order to investigate whether the HsCLS candidate protein indeed is able to synthesize CL, we purified mitochondria from the crd1Δ cells expressing the candidate HsCLS or harboring the empty vector and used these as enzyme source for in vitro CLS assays. Figure 4 shows that the expression of the putative HsCLS restores the formation of CL using CDP-dioleoyl [U-14C]-glycerol and dioleoyl-PG as substrates.

In conclusion, the growth phenotype complementation, the HPLC MS analysis and the CLS activity measurements of the different transformants demonstrate that the C20orf155 gene indeed encodes human CLS. The HsCLS ORF has been deposited in GenBank (accession number DQ386730). While this manuscript was under review, Lu and colleagues confirmed that this ORF indeed encodes HsCLS and is highly expressed in heart, skeletal muscle and liver [30].

**Characterization of HsCLS**

Since the substrate specificity of HsCLS has considerable implications for CL remodeling previous to or after the actual synthesis of the CL molecule, we investigated the substrate specificity for both PG and CDP-DAG species of the recombinant HsCLS enzyme. First, we optimized our assay conditions. Like CLS from various eukaryotes [8,15,31], the HsCLS enzyme has an alkaline pH optimum (figure 4), requires divalent cations for activity (figure 5) and has the highest
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Figure 4. pH profile of HsCLS. Reaction products were formed from 4 µM CDP-dioleoyl [U-14C]glycerol and 1 mM dioleoyl-PG by mitochondrial fractions of crd1Δ+HsCLS cells in 50 mM BTP-HCl buffer at different pH values.

Figure 5. TLC analysis of in vitro CLS assay. Reaction products were formed from CDP-dioleoyl [U-14C]glycerol and PG by mitochondrial fractions of crd1Δ+pYPGK18 cells (lanes 1, 3, 5, 7) and crd1Δ+HsCLS cells (lanes 2, 4, 6, 8) in the absence of divalent cations (lanes 1 and 2) or in the presence of 100 mM Co2+ (lanes 3 and 4) or 100 mM Mg2+ (lanes 5 and 6) or 100 mM Mn2+ (lanes 7 and 8).

activity in the presence of 100 mM divalent cations. Unlike the rat liver CLS [8], the HsCLS displayed similar activities with Co2+, Mn2+ and Mg2+ (figure 5). With the dioleoyl species of both substrates, the HsCLS showed maximal activity at 1 mM PG and 4.5 µM CDP-DAG resulting in specific enzyme activities of about 60 pmol/(min.mg protein) (data not shown). These data suggest that the HsCLS has a distinctly lower K_m value for CDP-DAG than for PG, as was reported for CLS from other eukaryotes [9,12,14,15]. Figure 6A shows that HsCLS was more active with dioleoyl and dilinoleoyl species of PG than with species with one or two palmitoyl groups as reported for the respective enzymes from plants [14,15]. Dimyristoyl-PG was a very poor substrate for the enzyme. The same PG species specificity as that shown in figure 6A was obtained when the CoCl_2 was substituted by MnCl_2. The CDP-DAG competition assays revealed that the specificity of HsCLS towards CDP-DAG species differed from that towards PG species, especially with regard to dimyristoyl substrate species. As depicted in figure 6B, CDP-dimyristoylglycerol as well as the species mixture from egg lecithin (primarily CDP-1-palmitoyl-2-oleoylglycerol) efficiently competed with the dioleoyl species for CL synthesis, whereas the dipalmitoyl species was distinctly less effective (figure 6B). Since the in vivo levels of CDP-DAG species containing medium-chain fatty acids are low when compared to long-chain fatty acids [32,33], the former are unlikely to represent the physiological substrates of CLS and contribute to CL synthesis, in spite of the preference of CLS in vitro. Interestingly, the substrate specificity of HsCLS towards PG and CDP-DAG is different, especially for dimyristoyl substrates. In contrast to CDP-dimyristoylglycerol - the best CDP-DAG substrate - dimyristoyl-PG is a very poor substrate for HsCLS. Of the more physiological substrates, CDP-DAG from
egg lecithin (primarily CDP-1-palmitoyl-2-oleoylglycerol) and CDP-dioleoylglycerol display similar activities. These data suggest that the incorporation of such mixed species into CL cannot be prevented by the properties of the HsCLS. In addition, the enzyme displayed no specificity for linoleoyl containing PG species when compared to the respective oleoyl containing species. Hence, the observed properties of the HsCLS underline the importance of remodeling mechanisms following CL synthesis in order to establish the typical fatty acid pattern of CL. Two remodeling mechanisms have been reported that can act upon the newly synthesized CL to obtain the specific linoleoyl-containing CL species. Experiments from Xu and colleagues elegantly show that different CL species can be efficiently remodeled into linoleoyl-containing CL species (predominantly tetralinoleoyl- and trilinoleoyl-oleoyl-CL) in the presence of labeled linoleoylphosphatidylcholine [17]. Ma and colleagues identified a monolyso-CL acyltransferase activity specific for oleoyl- and linoleoyl-CoA which is thought to be involved in the deacylation/reacylation cycle of CL [18].

An interesting observation is that the CL species in the wild type yeast strain are the same as those of the crd1Δ strain expressing the HsCLS. This suggests that the properties of human and yeast CLSs are very similar and thus appear to be evolutionarily conserved. This is in contrast with the large difference in CL composition of yeast and that of human tissues [34]. The high degree of linoleoyl content in human CL together with the evolutionary conserved substrate specificity of HsCLS suggests that suitable remodeling mechanisms have been developed as an additional system to obtain this more unsaturated composition.
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